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Further characterization of polysaccharides in seeds from *Ulmus glabra* Huds.

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The seeds of *Ulmus glabra* have been extracted with water at 90–100 °C yielding an extract which, by separation on a combination of anion exchange and gelfiltration columns, gave four different fractions, IA and B, and IIA and B. Structural studies showed that the first two can be designated as arabinogalactans due to the high content of arabinose and galactose, while the latter two fractions are predominantly polygalacturonides of the pectic type. Fractions IA and B contain arabinose both in the furanose and the pyranose form. All four fractions have the structural elements necessary for the binding of the arabinogalactan-Yariv artifical antigen.

INTRODUCTION

Bark of the common elm, *Ulmus glabra* Huds., was traditionally important both as a substitute for wheat when making bread in periods of poor harvest (Høeg, 1974), and as an agent for the treatment of wounds. The same uses are reported for the bark of *U. fulva* Mich. both in English (Grieve, 1976) and Red Indian tradition in Canada (Stark, 1981). The unripe seeds of *U. glabra* were quite sticky which could mean that similar compounds are present in the seeds and in the bark. A study of an extract from the seeds revealed the presence of polysaccharides of the pectic type (Barsett *et al.*, 1991*a*) which appeared to be somewhat different from the pectic type polysaccharides found in the inner bark of the tree itself (Barsett & Smestad Paulsen, 1991). It was therefore of interest to study the structure further and also investigate the reaction of the polysaccharides with the Yariv artificial antigen (Yariv *et al.*, 1967).

METHODS

General methods

For both preparative and analytical column chromatography, the fractions were monitored for the presence of carbohydrate colorimetrically (OD 487) by the phenol sulphuric acid method (Dubois *et al.*, 1956). For preparative chromatography, the peristaltic pump P-3 (Pharmacia, Uppsala, Sweden) and the Ultrorac 7000 fraction collector (LKB, Bromma, Sweden) were used. The quantitative monosaccharide compositions of the polymer fractions isolated were determined by gas capillary chromatography as *O*-TMS-methyl glycosides as previously described (Barsett *et al.*, 1991*a*). The results are given in Table 1.

Preparation of the polymer extract

Unripe seeds of *Ulmus glabra* Huds. collected in May 1985 on the University campus at Blindern were first treated with 80% ethanol to remove low molecular weight material and coloured matter. Afterwards, the seeds were extracted with distilled water at 100 °C for 4 h, filtered, centrifuged at 5000 rpm in a Sorvall® RC2-B automatic superspeed refrigerated centrifuge at 10 °C for 20 min, and the supernatant was dialyzed against distilled water and lyophilized.

Ion exchange chromatography on DEAE-Sepharose fast flow

The total polymer extract was applied to a DEAE-Sepharose fast flow column (40 cm × 5 cm i.d.) converted into the chloride form. The column was eluted at 1·7 ml/min by gradient elution using 0–1 M sodium chloride and 12 ml fractions were collected. This fractionation procedure gave two fractions, designated Fr.I and Fr.II, eluted by NaCl gradient in the ranges 0–0·2 M and 0·2–0·5 M, respectively.

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Table 1. Carbohydrate compositions[a] and molecular weight distribution of the different fractions of polysaccharides present in the seeds of *Ulmus glabra* Huds.

Monosaccharide	Fractions separated on the Mono P column				Fractions separated on Sepharose 6B			
	Fraction IA		Fraction IB		Fraction IIA		Fraction IIB	
	Orig.[b]	Peri.[c] oxid.	Orig.	Peri. oxid.	Orig.	Peri. oxid.	Orig.	Peri. oxid.
Arabinose	43	21	39	18	11	5	12	5
Rhamnose	9	5	10	9	14	8	13	7
Galactose	41	36	42	38	24	16	11	8
Galacturonic acid	7	0	9	0	51	15	64	25
Molecular weight distribution	20 000–28 000		17 000–22 000		180 000–300 000		30 000–70 000	

[a] Given as mg anhydrosugar/100 mg. Periodate results are based on the weight of the starting material.
[b] Original material.
[c] Periodate oxidised material.

Fractionation of Fr.I on a Mono P column using the fast protein liquid chromatography (FPLC) system

The anion exchange column, Mono P, was fitted to the FPLC system (Pharmacia) consisting of the units described earlier (Barsett & Smestad Paulsen, 1985). A 4 mg sample in 2 ml buffer was injected on to the column. The column was eluted at 0·75 ml/min, using 0–1 M sodium chloride in 15 mM phosphate–sodium chloride buffer (pH 7) (Fig. 1). Fractions of 1 ml were collected and tested for carbohydrate content as above.

Isolation of Fr.IA and Fr.IB on PBE 94™

For preparative purposes, isolation of Fr.IA and Fr.IB was performed on a Polybuffer exchanger 94™ column (20 cm × 5 cm i.d.) converted into the chloride form. The column was eluted at 1·6 ml/min by gradient elution using 0–1 M sodium chloride and 6·5 ml fractions were collected. The fractions were tested for carbohydrate content as described above.

Gelfiltration on Sepharose 6B

Separation of Fr.II and isolation of fractions Fr.IIA and Fr.IIB were performed on a Sepharose 6B gel-filtration column (100 cm × 2·5 cm i.d.). The column was eluted with 2% butanol in water at 1·3 ml/min and 5 ml fractions were collected. The fractions were monitored for carbohydrate content as described above.

Molecular weight estimation on Superose™ 12 column

The molecular weights of the different fractions were determined on a Superose™ 12 high performance gel filtration column using the FPLC system (Pharmacia).

The injection loop was 200 µl, and via this 800 µg samples were injected. The column was calibrated against dextrans (Pharmacia): Dextran T 500, lot 9301 MW 475.000, MN 153.000; Dextran T 250, lot 8374, MW 233.000, MN 123.000; Dextran 70, Fr.II - 4, MW 98.400, MN 84.800; Dextran B 512, Dex 40 T 8630 Fr. 7, MW 19.000, MN 18.500; Dextran PD T5558+7650 Fr. 3, MW 7600, MN 7150; and Dextran PD 5558 Fr. 18–20, MW 2280, MN 1900. The samples were filtered through a Millipore R filter with a pore size of 0.22 µm. The column was eluted at 0·5 ml/min, using 0·01 M sodium chloride with 2% butanol as eluent. Fractions of 0·25 ml were collected and tested for carbohydrate content as described above. The molecular weight relative to dextran, is given in Table 1.

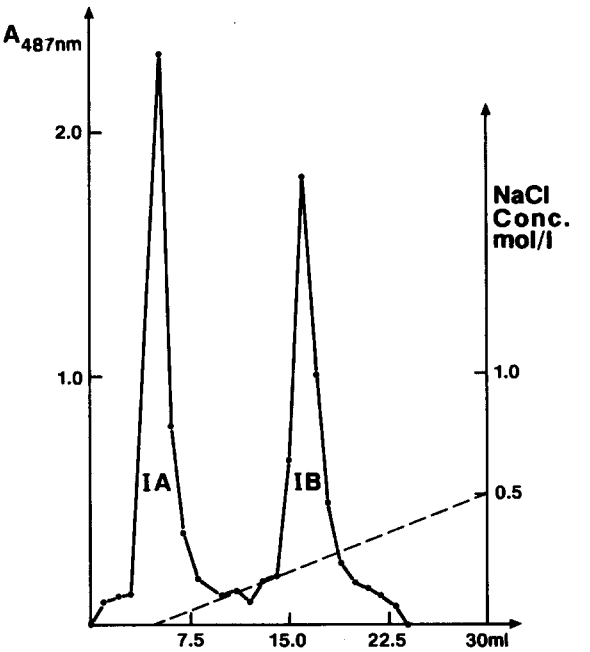

Fig. 1. Separation of Fr.I on the Mono P column in the FPLC system. (———) Carbohydrate profile; (- - - -) NaCl gradient.

Periodate oxidation

The sample (10 mg) was subjected to oxidation with 0·01 M $NaIO_4$ in 0·1 M NaOAc buffer pH 4 (10 ml) at 4°C in the dark. The consumption of periodate was followed spectrophotometrically at 223 nm (Aspinall & Ferrier, 1957), and the reaction terminated after 14 h by addition of ethylene glycol. Free aldehyde groups were reduced by $NaBH_4$. The solution was desalted, freeze-dried and subjected to methanolysis and analysis of monosaccharides by GC as described above. The results are given in Table 1.

Reduction of uronic acids in the polymers

Prior to reduction, the polysaccharides were de-esterified in 0·05 M NaOH for 2 h at 2°C, then neutralized to pH 4–5 with 0·5 M HCl (Saulnier *et al.*, 1988). 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate (Taylor & Conrad, 1972) (10 mol of CMC per carboxylic acid equivalent) was added, and the pH was kept at 4·75 with 0·01 M HCl for 2 h. $NaBH_4$ solution (250 mol of $NaBH_4$ per carboxylic acid equivalent) was then added over a 30 min period, while the pH was maintained at 7 with 1 M HCl (1-octanol was included as an anti-foaming agent). After 17 h, $NaBH_4$ solution (125 mol $NaBH_4$ per carboxylic acid equivalent) was added again over a 30 min period while the pH was maintained at 7 with 1 M HCl. The mixture containing the polysaccharide was then extensively dialysed, and then concentrated and freeze-dried.

Methylation analysis

The polysaccharides were methylated by the method described by Harris *et al.* (1984), and modified by Kvernheim (1987), using the lithium salt of methylsulphinyl carbanion (Blakeney & Stone, 1985). The partially methylated alditol acetates were analysed by GC–MS as previously described (Barsett *et al.*, 1991*a*). The results of the methylation analyses are given in Table 2.

Arabinogalactan-Yariv reagent crossed electrophoresis

These experiments were performed essentially as described by van Holst and Clarke (1986). A solution of 1% (w/v) agarose in 0·025 M Tris, 0·2 M glycine (pH 8·3) was heated to boiling. A sample (15·5 ml) was poured on to a preheated, level glass plate (10 cm × 10 cm). Nine wells (1·5 mm in diameter) were made in the gel. The top well was filled with 15 μl of bromophenol blue (0·2 mg/ml) in 50 mM Tris-HCl (pH 8·0) and the other wells were filled with 15 μl sample mixed with 0·8 μl of bromophenol blue (3 mg/ml). The first gel was run at 5 V/cm for about 55 min, or until the dye front moved 4 cm. The running buffer was 0·025 M Tris, 0·2 M glycine. After electrophoresis, the slice of a sample lane gel (1 cm × 5 cm) was moved to a Gel Bond film (Pharmacia) (6 cm × 5 cm) where the second gel (1 mm thick) was made of 1% (w/v) agarose in 0·025 M Tris, 0·2 M glycine and 90 μg/ml β-glucosyl-Yariv reagent (the last component was added after boiling). Electrophoresis at right angles to the direction of the first run was carried out at 10 V/cm for about 5 h. The non-precipitated Yariv reagent was removed by washing with 1% (w/v) NaCl followed by rinsing with distilled H_2O and the gels were dried in a warm air stream (Fig. 2).

Table 2. Glycosyl-linkage analysis of various polysaccharide fractions isolated from the seeds of *U. glabra* Huds.

Type of linkage		IA (%mol)	IB (%mol)	IIA (%mol)	IIB (%mol)
Ara*f*	T	11·2	11·7	5·1	6·7
Ara*p*	T	13·9	9·4		3·2
Ara*f*	1,3-	16·3	17·1	2·4	4·1
Ara*p*	1,4-	7·3	6·1	3·6	2·7
Rha*p*	T			1·2	1·6
	1,3-				tr
	1,2-	4·2		3·2	7·4
	1,2;1,3-				1·2
	1,2;1,4-	1·4	3·8	5·7	5·1
Gal*p*	T			4·5	0·7
	1,3-	7·2	5·1	6·8	1·2
	1,6-	3·1	2·0	3·7	0·4
	1,3;1,6-	32·0	38·4	10·5	9·4
GalA*p*[a]	1,4-	3·7	6·2	45·3	55·4
	1,4;1,3-			4·2	0·7
	1,4;1,2-			3·8	0·6

[a]Determined from methylated samples which had been carboxyl-reduced prior to methylation.

^{13}C-NMR

The sample (10 mg) was dissolved in D_2O (1 ml) and the spectra were recorded in a Varian XL-300 NMR.

RESULTS AND DISCUSSION

The seeds of *Ulmus glabra* Huds. were extracted as described previously (Barsett *et al.*, 1991*a*) except that the sample was heated at a temperature of 90–100°C for 4 h when preparing the crude polysaccharide material. This resulted in an extract appearing to have a different polysaccharide composition compared with the extract obtained when using a lower temperature for extraction of the seeds.

Separation of the polymer extract on the DEAE-Sepharose anion exchange column gave two fractions of approximately the same size. These fractions were eluted by salt gradient elution: the first, Fr.I, in the

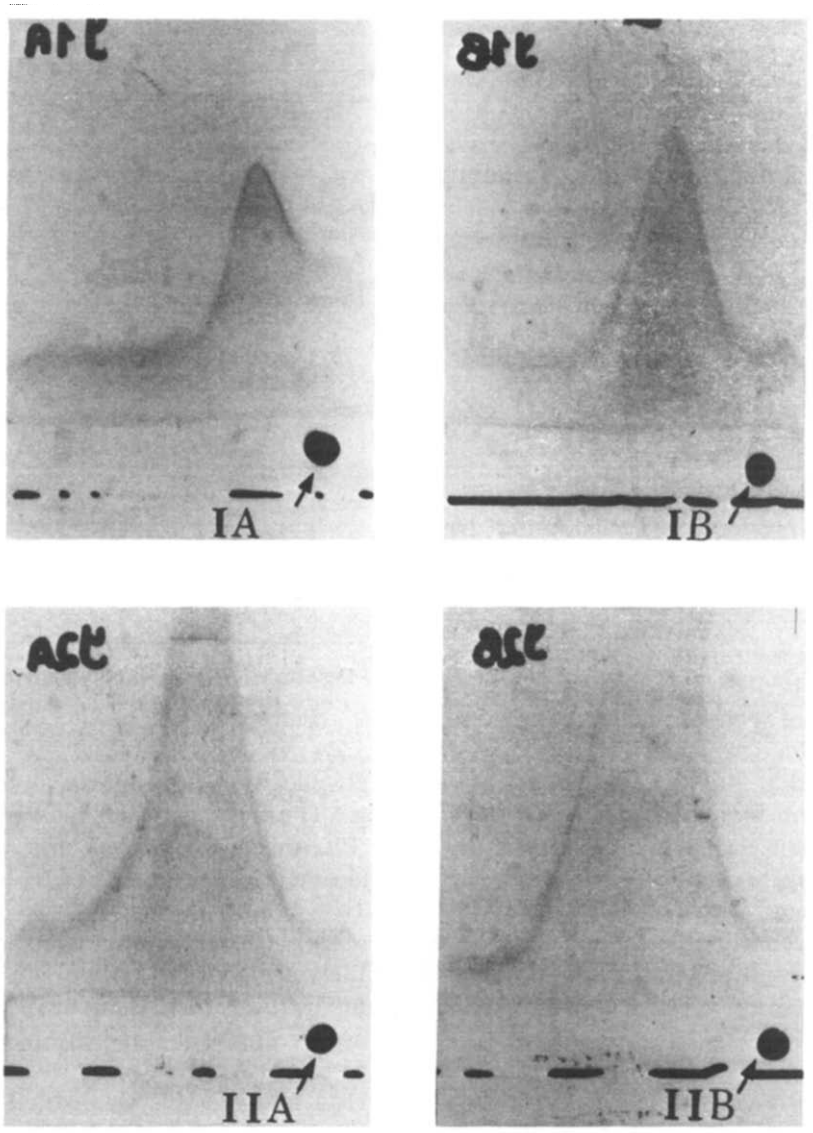

Fig. 2. Arabinogalactan-Yariv reagent crossed electrophoresis of IA (169 μg), IB (31·5 μg), IIA (90 μg) and IIB (105 μg). The application wells for the first dimension electrophoresis are marked with an arrow. The second dimension gel contained 90 μg/ml Yariv reagent. Details are given in the Methods section.

range 0–0·2 M NaCl; the other, Fr.II, between 0·2 and 0·5 M NaCl.

Further separation of Fr.I was achieved on the Polybuffer exchanger PBE 94 (Fig. 1 shows the separation obtained on the equivalent Mono P column in the FPLC system). Fraction I was divided into two distinct fractions—Fr.IA, which was eluted in the range 0–0·1 M NaCl, while Fr.IB was eluted between 0·15 and 0·2 M NaCl. Fraction II could not be separated further on an anion exchange column, but a separation was obtained by gel filtration giving two fractions in the molecular weight ranges given in Table 1.

The carbohydrate composition as well as the molecular weight ranges of the four fractions obtained are given in Table 1. Fractions IA and IB appear to have quite similar carbohydrate compositions, with little difference in the MW. As for fractions IIA and IIB, the monomer compositions are also quite similar, but the MW differ by a factor of approximately 5.

Fractions IA and IB can both be looked upon as being arabinogalactans, as arabinose and galactose constitute more than 70% of the carbohydrate present. Both fractions were subjected to periodate oxidation, and the results in Table 1 indicated that all the galacturonic acid present was 1,4 linked, while approximately half of the arabinose present might have adjacent hydroxyl groups, thus being susceptible to oxidation. Little of the rhamnose and galactose was destroyed by this treatment.

Fractions IIA and IIB, with more than half of the sugar moiety as galacturonic acid, indicated that some of the galacturonic acid residues were branchpoints as

approximately one-third of this sugar remained after oxidation. Approximately half of the arabinose and rhamnose was attacked and approximately one-third of galactose in both fractions IIA and IIB.

Linkage analyses of the monomers present in the different polysaccharide fractions were determined both before and after reduction of the uronic acid present. The results are summarized in Table 2. A slight degree of undermethylation might have occurred as there is a discrepancy between the number of endgroups and branchpoints in the results obtained. In Fr.IA and IB the arabinose was found to be present both in furanose and pyranose form. These fractions were subjected to weak acid hydrolysis (Smestad *et al.*, 1975) and the results showed that approximately half of the arabinose units was lost, while all the other sugars were still present in the same proportions. This also indicated that the arabinose was present both in the furanose and pyranose form in equal proportions. The furanosides are endgroups and 1,3-linked units, while the pyranosides also are present as endgroups and in addition as 1,4-linked units. The main part of the galactose represents branchpoints in the polymer linked at both positions 3 and 6. Those units not present as branchpoints are linked either at position 3 or at position 6. The few rhamnose units present appear to be 1,2-linked with branches at C4, while the galacturonic acid is 1,4-linked. The types of linkages are quite common in many of the arabinogalactans of pectic substances which have been described in the literature (Darvill *et al.*, 1980). The ^{13}C NMR spectra of Fr.IA and IB were identical for the two fractions. The assignment of some of the signals is given in Table 3. The presence of arabinose both as furanoside and pyranoside residues was verified by the presence of the signals near 112 and 102 ppm (Joseleau *et al.*, 1977; Blake *et al.*, 1983). The heights of the signals are approximately equal, indicating the presence of the two forms in the same magnitude. This supports the results from the weak acid hydrolysis experiment described above and also the results from the methylation studies in Table 2.

The arabinofuranoside thus appears to be present as α-linked units, and the arabinopyranoside as β-linked. The signals around 64 ppm verify that C5 of the arabinofuranose are not linked via this position to another sugar unit (Joselaeu *et al.*, 1977; Saulnier *et al.*, 1988). The galactose units appear to be β-linked (Blake *et al.*, 1983; Bock *et al.*, 1984; Saulnier *et al.*, 1988).

Table 3. Assignments of signals from ^{13}C-NMR of Fr.IA and IB

Signals	Structural element
111·9–112·1 ppm	C1 of α-L-arabinofuranosides
105·9 ppm	C1 of β-D-galactopyranosides
102·1 ppm	C1 of β-L-arabinopyranosides
64·0–64·2 ppm	C5 of α-L-arabinofuranosides not involved in a linkage

Crossed electrophoresis using the Yariv artificial antigen (Yariv *et al.*, 1967) in the second direction gave precipitation arches for both fractions as shown in Fig. 2. Several arabinogalactans do precipitate with this reagent, and common for most of them is the presence of galactose being both 3- and 6-linked. The arabinose is mainly present as endgroups. The type of binding between the Yariv reagent and the polysaccharides is not known (Clarke *et al.*, 1979; Fincher *et al.*, 1983; Bacic *et al.*, 1988).

The Fr.II obtained when the seeds were extracted at 100 °C are not identical to the one obtained previously (Barsett *et al.*, 1991*a*), the main difference being that the glucuronic acid is now absent. The structural features are otherwise fairly similar. The reason for the differences may be due to the fact that partial hydrolysis may take place when using extraction and isolation procedures involving heating at 100 °C (Clarke *et al.*, 1979). These acidic polysaccharides consist mainly of a galacturonan backbone, interspersed with a few rhamnose units. The backbones have branchpoints at C2 or C3 of some of the galacturonic acid residues; this differs from what is found for PBI, PBII and PBIII from the inner bark of *U. glabra* (Barsett *et al.*, 1991*b*). The periodate oxidation results (Table 1) also indicate a higher degree of branching for the seed polymers. The low degree of oxidation may also be due to underoxidation caused by the formation of hemiacetals (Painter & Larsen, 1970).

Fractions IIA and IIB did precipitate with the Yariv reagent in crossed electophoresis (Fig. 2). It is interesting to note that two of the acidic polysaccharides isolated from the inner bark of *U. glabra*, PBII and PBIII, did not precipitate with the Yariv reagent (Barsett *et al.*, 1991*b*). The structural features of PBI appear to be more similar to those found in the seeds as 3,6-linked galactose is present in these three polysaccharides and almost absent in PBII and PBIII.

It was impossible to dissolve the Fr.II polymers in sufficient concentration in order to record NMR spectra of these fractions in any of the different solvents tested, e.g. DMSO, tetramethylurea at high temperatures.

CONCLUSION

The seeds of *U. glabra* contain pectic substances which can be extracted by boiling water. The material obtained γ_1 will probably undergo partial hydrolysis during the isolation and fractionation procedure, and can be separated into four different subfractions, Fr.IA and IB, which are predominantly arabinogalactans, and Fr.IIA and IIB, which are predominantly poly-

galacturonans. They all have the structural features necessary for precipitation with the Yariv antigen.

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REFERENCES

Aspinall, G. O. & Ferrier, R. J. (1957). *Chem. Ind.*, 1216

Bacic, A., Gell, A. C. & Clarke, A. E. (1988). *Phytochemistry.*, **27**, 679–84.

Barsett, H. & Smestad Paulsen, B. (1985). *J. Chromatogr.*, **329**, 315.

Barsett, H. & Smestad Paulsen, B. (1991). *Carbohyd. Polym.*, in press.

Barsett, H., Haraldstad, K.-A. & Smestad Paulsen, B. (1991*a*). *Acta Pharm. Nord.*, **3**, 87–90.

Barsett, H., Aslaksen, T., Bratvedt, A & Smestad Paulsen, B. (1991*b*). *Carbohyd. Polym.*, in press.

Blake, J. D., Clarke, M. L. & Jansson, P. E. (1983). *Carbohydr. Res.*, **115**, 265–72.

Blakeney, A. B. & Stone, B. A. (1985). *Carbohydr. Res.*, **140**, 319.

Bock, K., Pedersen, C. & Pedersen, H. (1984). *Adv. Carb. Chem. Biochem.*, **42**, 193–225.

Clarke, A. E., Anderson, R. L. & Stone, B. A. (1979). *Phytochemistry*, **18**, 521–40.

Darvill, A., McNeil, M., Albersheim, P. & Delmer, D. P. (1980). In *The Biochemistry of Plants, Vol. 1*, ed. N. E. Tolbert. Academic Press, New York, pp. 101–16.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Anal. Chem.*, **28**, 350.

Fincher, G. B., Stone, B. A. & Clarke, A. E. (1983). *Ann. Rev. Plant Physiol.*, **34**, 47–70.

Grieve, M. (1976). *A Modern Herbal* (2nd edn). Penguin Books, Harmondsworth, UK.

Harris, P. J., Henry, R. J., Blakeney, A. B. & Stone, B. A. (1984). *Carbohydr. Res.*, **127**, 59.

Høeg, O. A. (1974). *Planter og tradisjon—Floraen i levende tale og tradisjon i Norge 1925–1973.* Universitetsforlaget, Oslo, p. 651.

van Holst, G.-J. & Clarke, A. E. (1986). *Plant Physiol.*, **80**, 786–9.

Joseleau, J.-P., Chambat, G., Vignon, M. & Barnoud, F. (1977). *Carbohydr. Res.*, **58**, 165–75.

Kvernheim, A. L. (1987). *Acta Chem. Scand.*, **B14**, 150.

Painter, T. J. & Larsen, B. (1970). *Acta Chem. Scand.*, **24**, 813.

Saulnier, L., Brillouet, J.-M. & Joseleau, J.-P. (1988). *Carbohydr. Res.*, **182**, 73–8.

Smestad, B., Haug, A. & Myklestad, S. (1975). *Acta Chem. Scand.*, **B29**, 337–40.

Stark, R. (1981). *Guide to Indian Herbs.* Hancock House Publishers Ltd, Vancouver.

Taylor, R. L. & Conrad, H. E. (1972). *Biochemistry*, **11**, 1383.

Yariv, J., Lis, H. & Katchalski, E. (1967). *Biochem. J.*, **105**, 1C-2C.